Ceramide Regulates Lipopolysaccharide-Induced Phosphatidylinositol 3-Kinase and Akt Activity in Human Alveolar Macrophages[1]

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The phosphatidylinositol (PI) 3-kinase pathway is an important regulator of cell survival. In human alveolar macrophages, we found that LPS activates PI 3-kinase and its downstream effector, Akt. LPS exposure of alveolar macrophages also results in the generation of ceramide. Because ceramide exposure induces apoptosis in other cell types and the PI 3-kinase pathway is known to inhibit apoptosis, we determined the relationship between LPS-induced ceramide and PI 3-kinase activation in alveolar macrophages. We found that ceramide exposure activated PI 3-kinase and Akt. When we blocked LPS-induced ceramide with the inhibitor D609, we blocked LPS-induced PI 3-kinase and Akt activation. Evaluating cell survival after ceramide or LPS exposure, we found that blocking PI 3-kinase induced a significant increase in cell death. Because these effects of PI 3-kinase inhibition were more pronounced in ceramide- vs LPS-treated alveolar macrophages, we also evaluated NF-κB, which has also been linked to cell survival. We found that LPS, to a greater degree than ceramide, induced NF-κB translocation to the nucleus. As a composite, these studies suggest that the effects of ceramide exposure in alveolar macrophages may be very different from the effects described for other cell types. We believe that LPS induction of ceramide results in PI 3-kinase activation and represents a novel effector mechanism that promotes survival of human alveolar macrophages in the setting of pulmonary sepsis. ***The Journal of Immunology*****, 2001, 167: 5977–5985.**

Lipopolysaccharide plays a pivotal role in the innate immune response to Gram-negative bacteria (1, 2). LPS signaling is initiated by an interaction between LPS and LPS-binding protein (LBP),[3] allowing binding to CD14 and association with at least one other cell membrane receptor, which contains an intracellular signaling domain (3–5). LPS signaling has been specifically linked to a member of the Toll-like receptor (TLR) family, TLR 4, both in vitro and in murine knockout models (6–11). The currently defined proximal signaling pathways activated by LPS downstream of the TLRs are similar to those used by IL-1 or IL-18: myeloid differentiation protein (MyD88) → IL-1 receptor-associated kinase → TNF receptor-associated factor 6, leading to the activation of NF-κB (12). Activation of phosphatidylinositol (PI) 3-kinase (a dual protein and lipid kinase) is also a proximal event in LPS signaling.

We have recently shown that in alveolar macrophages, LPS activates PI 3-kinase and, downstream of PI 3-kinase, Akt (13, 14). Akt regulates a diverse array of cellular processes, including having a profound effect on cell survival (15–19). Constitutive activation of either PI 3-kinase or Akt blocks apoptosis induced by c-Myc, UV radiation, TGF-β, and Fas (20–24). In addition, Akt can significantly prolong survival in a murine model of oxidant-induced injury (25). Akt is activated following PI 3-kinase recruitment to the inner surface of the plasma membrane. Membrane-associated PI 3-kinase catalyzes the transfer of ATP to the D-3 position of the inositol ring of membrane-localized phosphoinositides (26). This results in the production of a number of bioactive species including PI 3 phosphate (PI_3P), PI 3,4 phosphate ($PI_{3,4}P$), and PI 3,4,5 phosphate ($PI_{3,4,5}P$). Both $PI_{3,4}P$ and $PI_{3,4,5}P$ are nominally absent in most unstimulated cells and increase dramatically following PI 3-kinase activation. The production of $PI_{3,4,5}P$, especially, results in the recruitment of 3-phosphoinositide-dependent kinase (PDK-1), a kinase with multiple downstream substrates, including Akt (27).

Akt, like PDK-1, is recruited to membrane-bound D3 phosphorylated PIs (D3 PPIs) by its Pleckstrin homology domain. Binding of Akt to D3 PPIs results in a conformational change allowing phosphorylation by PDK-1 (on threonine 308 in the activation loop) and an activating phosphorylation at serine 473 within the hydrophobic motif at the kinase tail (16). Activation of Akt results in the phosphorylation of a number of substrates that have potential importance in LPS signaling (GSK-3, Bad, caspase 9, Forkhead transcription factors, Raf-1, IκB kinase, phosphodiesterase-3B and endogenous nitric oxide synthase) (15, 16, 26, 28). Phosphorylation of these proteins by Akt results in either activation or inactivation, depending on the substrate. Inactivation of some of the proapoptotic factors, caspase 9, Bad, GSK-3, and the

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[3] Abbreviations used in this paper: LBP, LPS-binding protein; TLR, Toll-like receptor; OPA, ophthalaldehyde; MyD88, myeloid differentiation protein; PDK-1,3-phosphoinositide-dependent kinase; PC-PLC, phosphatidylcholine-specific phospholipase C; PI, phosphatidylinositol; D3 PPI, D3 phosphorylated PI; PI_3P, PI 3 phosphate; EthD-1, ethidium homodimer-1.

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Forkhead family of transcription factors, are central to Akt's role in cell survival (19, 29–33).

Unlike activation of PI 3-kinase or Akt, which are associated with enhanced cell survival, activation of the sphingomyelin hydrolysis pathway has been linked to apoptosis (34). Ceramide, a sphingomyelin hydrolysis product, is strongly associated with apoptotic cell death and is triggered within minutes via the action of neutral and acid sphingomyelinases (35–38). Ceramide can also be generated by de novo synthesis initiated with the condensation of serine and palmitoyl-CoA catalyzed by serine palmitoyltransferase and ending with the conversion of dihydroceramide to ceramide by dihydroceramide reductase (36). However, this process is slow, taking up to several hours. Apoptosis, induced by multiple factors including TNF, CD95/Fas, ionizing radiation, UV light, heat shock, IL-3 withdrawal, and sodium arsenate treatment, has been linked to ceramide generation. Thus, ceramide has been proposed as a universal feature of apoptosis (39–41).

LPS has been demonstrated to increase ceramide content in diverse systems (39–46). LPS induction of ceramide in alveolar macrophages results from sphingomyelin hydrolysis, an effect downstream of phosphatidylcholine-specific phospholipase C (PC-PLC) activation in alveolar macrophages (43). In this study, we make use of the inhibitor D609 (previously shown to block LPS-induced ceramide) to show that LPS-induced ceramide plays a role in activation of the PI 3-kinase pathway. We have recently demonstrated that LPS activates the PI 3-kinase pathway in alveolar macrophages (14, 47, 48). In the present study, we hypothesized that LPS-induced ceramide may be an important effector in LPS-induced activation of the PI 3-kinase pathway in alveolar macrophages. Our results demonstrate that ceramide (and LPS-induced ceramide) can activate PI 3-kinase and Akt in alveolar macrophages and that this is linked to maintenance of cell viability. We found that the proapoptotic effect of ceramide is observed in these cells only if the PI 3-kinase pathway is inhibited.

Materials and Methods

Materials

Chemicals were obtained from Sigma-Aldrich (St. Louis, MO). Protease inhibitors were obtained from Boehringer Mannheim (St. Louis, MO). Ethidium homodimer was obtained from Molecular Probes (Eugene, OR). GammaBind Sepharose was obtained from Amersham Pharmacia Biotech (Piscataway, NJ). Nitrocellulose and ECL Plus were obtained from Amersham (Arlington Heights, IL). SuperSignal West Femto was obtained from Pierce (Rockford, IL). Abs were obtained from various sources: Abs to extracellular signal-related kinase, Akt, and GSK-3 from Santa Cruz Biotechnology (Santa Cruz, CA); PDK-1 from Upstate Biotechnology (Waltham, MA); phosphorylation-specific Abs to extracellular signal-related kinase from Sigma-Aldrich; all other phosphorylation-specific Abs from Cell Signaling (Beverly, MA); and Ab to the p85 regulatory unit of PI 3-kinase from Calbiochem (San Diego, CA). PI 3 phosphate was obtained from Sigma-Aldrich. D-Erythro-sphingosine, D,L-erythro-dihydrosphingosine (sphinganine), and D-erythro-C20-sphingosine were purchased from Matreya (Pleasant Gap, PA). Ophthalaldehyde (OPA) was purchased from Sigma-Aldrich. HPLC reagents were obtained from Fisher Scientific (Pittsburgh, PA).

Isolation of human alveolar macrophages

Alveolar macrophages were obtained from bronchoalveolar lavage as previously described (49). Briefly, normal volunteers with a lifetime nonsmoking history, no acute or chronic illness, and no current medications underwent bronchoalveolar lavage. The lavage procedure used five 25-ml aliquots of sterile, warmed saline in each of three segments of the lung. The lavage fluid was filtered through two layers of gauze and centrifuged at $1500 \times g$ for 5 min. The cell pellet was washed twice in HBSS without Ca^{2+} and Mg^{2+} and suspended in complete medium, RPMI 1640 tissue culture medium (Life Technologies/BRL, Gaithersburg, MD) with 100 ng/ml LBP (a gift from P. Tobias, The Scripps Research Institute, La Jolla, CA) and added gentamicin (80 μg/ml). Differential cell counts were determined using a Wright-Giemsa stained cytocentrifuge preparation. All cell preparations had between 90 and 100% alveolar macrophages. This study was approved by the Committee for Investigations Involving Human Subjects at the University of Iowa (Iowa City, IA).

Isolation of whole cell extracts

Alveolar macrophages were cultured for various times. Whole cell protein was obtained by lysing the cells on ice for 20 min in 500 μl of lysis buffer (0.05 M Tris (pH 7.4), 0.15 M NaCl, 1% Nonidet P-40, 0.5 M PMSF, 50 μg/ml aprotinin, 10 μg/ml leupeptin, 50 μg/ml pepstatin, 0.4 mM sodium orthovanadate, 10 mM sodium fluoride, and 10 mM sodium pyrophosphate). The lysates were then sonicated for 20 s and spun at $15{,}000 \times g$ for 10 min, and the supernatant was saved. Protein determinations were made using a protein measurement kit from Bio-Rad (Hercules, CA).

Isolation of cytoplasmic and membrane proteins

Alveolar macrophages were cultured for various times in different conditions. Cell pellets were suspended in 200 μl of lysis buffer (see whole cell protocol) without Tween 20, pulse sonicated ($1 \text{ s} \times 20$) on ice, and then spun at $100{,}000 \times g$ (55,000 rpm) for 1 h. The supernatant (cytoplasmic fraction) was saved at −70°C. The membrane pellet was resuspended in 100 μl of lysis buffer with 1% Tween 20 and sonicated for 5 s on ice. After 20 min, cell debris was removed (14,000 rpm for 10 min) and the supernatant was saved. Western blot analysis was performed as described below.

Immunoprecipitation

Alveolar macrophages were cultured in complete medium with or without LPS (100 ng/ml; Sigma-Aldrich). After isolating protein, 200–600 μg from each sample was removed for immunoprecipitation. The samples were cleared by incubating for 2 h with 1 μg/sample rabbit IgG and 10 μl/sample GammaBind Sepharose. After centrifuging, the supernatants were transferred to a tube containing 3 μg/sample Ab bound to GammaBind Sepharose and rotated at 4°C overnight. The beads were subsequently washed three times with high-salt buffer (0.5 M Tris (pH 7.4), 0.50 M NaCl, and 1% Nonidet P-40) and three times with lysis buffer without protease inhibitors. The immunoprecipitated complexes were used either for Western blot analysis or for kinase activity assays.

Western blot analysis

Western blot analysis for the presence of particular proteins or for phosphorylated forms of proteins was performed on whole cell or cytosol/membrane proteins from alveolar macrophage experiments. Protein (50–100 μg) was mixed 1/1 with 2× sample buffer (20% glycerol, 4% SDS, 10% 2-ME, 0.05% bromophenol blue, and 1.25 M Tris (pH 6.8); all chemicals from Sigma-Aldrich) and loaded onto a 10% SDS-PAGE gel and run at 30 mA for 3 h. Cell proteins were transferred to nitrocellulose overnight at 30 V. Equal loading of the protein groups on the blots was evaluated using Ponceaus S (Bio-Rad), a staining solution designed for staining proteins on nitrocellulose membranes. The nitrocellulose was then blocked with 5% milk in TTBS (Tris buffered saline with 0.1% Tween 20) for 1 h, washed, and then incubated with the primary Ab at dilutions of 1/500 to 1/2,000 overnight. The blots were washed four times with TTBS and incubated for 1 h with HRP-conjugated anti- IgG Ab (1/5,000 to 1/20,000). Immunoreactive bands were developed using a chemiluminescent substrate, ECL Plus (Amersham Pharmacia Biotech) or SuperSignal West Femto (Pierce). An autoradiograph was obtained, with exposure times of 10 s to 2 min.

In vivo phosphorylation of Akt

Alveolar macrophages were labeled with 1.25 mCi of $^{32}P_i$/group (NEN Life Science Products, Boston, MA) in phosphate-free RPMI 1640 without serum for 3 h at 37°C. The cells were harvested and placed in RPMI 1640 with 100 ng/ml LBP and treated with D609 for 30 min. After the D609 incubation, the cells were stimulated with LPS for various times at 37°C. The cells were harvested, resuspended in lysis buffer (1% Nonidet P-40, 1% sodium deoxycholate, 0.1% SDS, 0.15 M NaCl, 0.01 M Na_3P0_4 (pH 7.2), 2 mM Na_3VO_4, 1 μM okadaic acid, 100 μg/ml phenylmethylsulfonyl fluoride, 50 μg/ml aprotinin, 10 μg/ml leupeptin, and 50 μg/ml pepstatin), and sonicated. Akt was immunoprecipitated from the lysate and the sample was separated on a 10% SDS-PAGE discontinuous gel as described above. The gel was dried and an autoradiograph was obtained.

Ceramide determination by HPLC

Ceramide (an *N*-acylated sphingosine) was extracted from cells and resolved from sphingosine using TLC before an acid hydrolysis step (converting it to sphingosine), before derivatization and analysis by HPLC as described (50). Sphingosine (0.25–1 mg of protein per sample) plus 200

pM D-erythro-C20-sphingosine (an internal standard) were extracted according to the method of Bligh and Dyer (51). The chloroform layer was isolated and dried under nitrogen gas. The dried extracts were resuspended in 0.33 ml of chloroform and 0.66 ml of 0.1 M KOH in methanol and incubated at 37°C for 1 h. The samples were rinsed with 1 ml of chloroform and 1 ml of 1.0 M NaCl. The chloroform phase was washed with NaCl and dried under nitrogen gas. Orthophthalaldehyde derivatives were prepared by dissolving the dried samples in 50 ml of methanol, followed by the addition of 50 ml of OPA reagent (5 mg of OPA in 100 ml of ethanol, 9.9 ml of 3% boric acid, and 5 ml of 2-ME), incubated at room temperature for 5 min, diluted with methanol/water (94/6 v/v), and quantitated by HPLC (52). Orthophthalaldehyde derivatives were separated on a Beckman Ultrasphere C-18 column (Beckmam Coulter, Fullerton, CA), with methanol/water (94/6 v/v) mobile phase at a rate of 1 ml/min. The derivatives were detected using a Thermoseparation Products Spectra System FL3000 fluorescence detector (Thermo Separation Products, San Jose, CA) at 340-nm excitation and 454-nm emission wavelengths.

PI 3-kinase activity assay

After culture, whole cell lysates were obtained and PI 3-kinase was immunoprecipitated using an Ab to the p85 regulatory subunit of PI 3-kinase. Activity was assayed by measuring the formation of $PI_{3,4}[^{32}P]$phosphate (53, 54). After overnight incubation with Ab-coated beads (see immunoprecipitation methods), the bound protein was washed three times with buffer I (phosphate buffered saline containing 1% Nonidet P-40 and 100 μM Na_3VO_4) and then three times with buffer II (100 mM Tris-HCl (pH 7.5), 500 mM LiCl, and 100 μM Na_3VO_4), and finally three times with buffer III (Tris-HCl (pH 7.5), 100 mM NaCl, 1 mM EDTA, and 100 μM Na_3VO_4). After washing, immunoprecipitates were resuspended in 50 μl of buffer III with the addition of 10 μl of 100 mM $MgCl_2$ and 10 μl of PI_4P (2 μg/ml). The samples sat at room temperature for 5 min before the addition of 10 μl of ATP (440 μM ATP with 30 uCi/10 μl of [γ-^{32}P]ATP). The samples were then shaken at room temperature for 10 min. The reaction was stopped by the addition of 20 μl of 8N HCl and 160 μl of chloroform/methanol 1/1. The lipids were extracted by standard methods, dried down, resuspended in 20 μl of chloroform/methanol 1/1, and separated on thin-layer silica gel plates (pretreated with 10% w/v potassium oxalate) in a solvent system of chloroform/methanol/water/NH_4OH (60/47/11/2.2 v/v/v/v). Incorporation of ^{32}P into $PI_{3,4}P$ was detected by autoradiography and activity was quantified on a Bio-Rad Molecular Imager FX.

Isolation of nuclear extracts and EMSAs

Alveolar macrophages were cultured for 3 h with LPS or ceramide. The nuclear pellets were prepared by resuspending cells in 0.4 ml of lysis buffer (10 mM HEPES (pH 7.8), 10 mM KCl, 2 mM MgCl2, and 0.1 mM EDTA), placing them on ice for 15 min, and then mixing them vigorously after the addition of 25 μl of 10% Nonidet P-40. After a 30-s centrifugation (16,000 $\times$ *g* at 4°C), the pelleted nuclei were resuspended in 50 μl of extraction buffer (50 mM HEPES (pH 7.8), 50 mM KCl, 300 mM NaCl, 0.1 mM EDTA, and 10% glycerol) and incubated on ice for 20 min. Nuclear extracts were stored at 70°C. The DNA binding reaction (EMSA) was done at room temperature in a mixture containing 5 μg of nuclear proteins, 1 μg of poly(d(I:C)), and 15,000 cpm of ^{32}P-labeled double-stranded oligonucleotide probe for 30 min. The samples were fractionated through a 5% polyacrylamide gel in 1$\times$ TBE (6.05 g/l Tris base, 3.06 g/l boric acid, and 0.37 g/l EDTA-Na_2·H_20). Sequence of the nucleotide was 5′-AGTT GAGGGGATTTTCCCAGGC-3′ (NF-κB).

Cell survival assay

Alveolar macrophages were cultured for 24 h under various conditions. Cell viability was assessed using an ethidium homodimer-1 (EthD-1) from Molecular Probes. Cells were cultured at 1 million cells/ml in 6-well tissue culture plates. At the end of the 24-h incubation, EthD-1 was added directly to the cultures (final concentration = 16 μM). The cultures were placed back in the incubator for 30 min and then assessed for ethidium entry into the cells by fluorescent microscopy and fluorescent plate reader (dead cells have brightly staining nuclei). Cells were then killed by treatment with 0.1% saponin, plate reader analysis was reperformed, and percent live cells were calculated: ((saponin cells − EthD-1 medium) − (sample cells − EthD-1 medium)/(saponin cells − EthD-1 medium)) $\times$ 100.

Statistical analysis

Statistical analysis when appropriate was determined using Student's *t* test. Values of $p < 0.05$ were considered significant.

Results

LPS activates the PI 3-kinase pathway in alveolar macrophages via generation of ceramide

We initially wanted to confirm that LPS activates the PI 3-kinase pathway and to determine whether that activation was linked to LPS-generated ceramide. To link LPS-induced PI 3-kinase activity and LPS-generated ceramide, we made use of the compound D609. D609 is a well-documented inhibitor of PC-PLC and we have shown that it inhibits LPS-generated ceramide (55, 56). Our previous work, combined with the fact that D609 inhibits the activity of exogenously added PC-PLC, leads us to believe that in our cells, D609 acts directly on PC-PLC, resulting in a block of LPS-induced ceramide generation. In the present studies, we found that D609 blocked the LPS-induced activation of PI 3-kinase as demonstrated by a decrease in the amount of PI_4P that is phosphorylated in an in vitro kinase activity assay (Fig. 1*A*). The initial event following the generation of D3 PPIs is the recruitment to the membrane of the main PI 3-kinase effector kinase, PDK-1. We found that D609 blocked the membrane recruitment of PDK-1 (see Fig. 1*B*). These observations suggest that the activation of PI 3-kinase by LPS is downstream of LPS-induced ceramide.

We next determined whether D609 blocks LPS-induced phosphorylation of kinases that are downstream of PI 3-kinase and PDK-1. We evaluated these events using phosphorylation-specific Abs or in vivo phosphorylation. Activation of the PI 3-kinase pathway has been shown to result in phosphorylation of Akt in the activation loop and to be essential for activation on a serine in the catalytic domain (serine 473). GSK-3 is a constitutively active kinase that can be inactivated via phosphorylation by Akt. We have shown previously that LPS-induced Akt activation results in the phosphorylation and inactivation of GSK-3 (serine 9) (14). In this study, we found that LPS-induced phosphorylations of Akt and GSK-3 were blocked by D609 (see Fig. 1*C*), demonstrating a link to LPS-induced ceramide. To confirm the validity of the Western blot findings, we performed an in vivo phosphorylation assay for Akt. Alveolar macrophages were labeled with ^{32}P and then treated with LPS, with and without D609. Whole cell protein was obtained, and Akt was immunoprecipitated and evaluated by SDS-PAGE. This experiment demonstrates complete inhibition of the in vivo phosphorylation of Akt when LPS-induced ceramide is blocked (see Fig. 1*D*). We have previously shown that Akt activation and GSK-3 inactivation are downstream of PI 3-kinase (14). These studies show that multiple kinases in the PI 3-kinase pathway may be downstream of LPS-induced ceramide.

Addition of PC-PLC to alveolar macrophages results in activation of PI 3-kinase

Because LPS induces ceramide via activation of a PC-PLC, we next evaluated the effect of increased PC-PLC activity on PI 3-kinase activation. In this experiment, we treated alveolar macrophages with a PC-PLC isolated from *Bacillus cereus* and evaluated PI 3-kinase activity (Fig. 2). We found increased activity at multiple time points (Fig. 2*A*). In Fig. 2*B* we confirmed the inhibitory activity of D609 by showing that it blocked PC-PLC activation of PI 3-kinase. These studies show that a PC-PLC (and the ceramide generated by PC-PLC (Fig. 3)) can trigger activation of the PI 3-kinase pathway in alveolar macrophages.

LPS exposure results in the generation of ceramide in alveolar macrophages

We have previously demonstrated that LPS increases amounts of ceramide in alveolar macrophages (43). In that study, the ceramide levels were evaluated using a two-step assay involving the in vitro

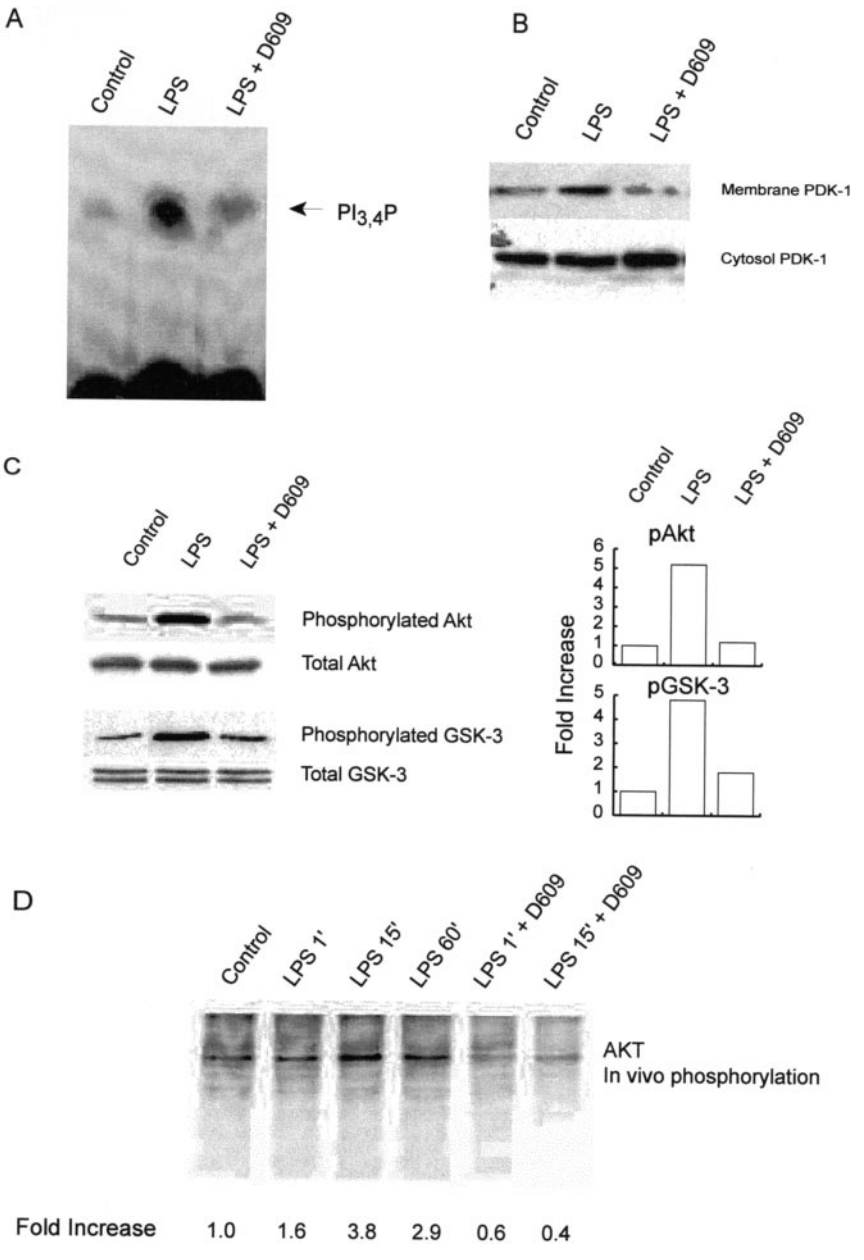

FIGURE 1. LPS activates the PI 3-kinase pathway via induction of ceramide. *A*, Alveolar macrophages were treated with LPS (100 ng/ml) with and without 10 μM D609 (a blocker of LPS-induced ceramide) for 10 min. Whole cell protein was obtained and PI 3-kinase was immunoprecipitated from 400 μg of the lysates using an Ab specific for the p85 regulatory unit. Kinase activity was determined by evaluating phosphorylation of PI_4P by the immunoprecipitated PI 3-kinase. The resulting $PI_{3,4}P$ was separated in a TLC system and an autoradiogram was obtained. *B*, Evaluation of PDK-1 translocation was conducted in alveolar macrophages. Cells were stimulated with LPS (100 ng/ml) with and without D609 for 5 min, and then cytosolic and membrane fractions were obtained. Western blot analysis was performed for PDK-1. Immunoreactive bands were visualized with chemiluminescence and autoradiograms were obtained with exposure times of 10 s to 5 min. *C*, Alveolar macrophages were treated with LPS (100 ng/ml) with and without D609 (10 μM) for 15 min. Whole cell protein was obtained and Western blot analysis was performed using Abs specific for Akt phosphorylated on serine 473 and GSK-3 phosphorylated on serine 21 in GSK-3 α and serine 9 in GSK-3 β. Equal loading of the blots was demonstrated by stripping the blot and reprobing with an Ab for Akt or GSK-3 (α and β isoforms). Primary Ab concentrations of 1/500 and secondary Ab concentrations of 1/5000 were used. Immunoreactive bands were visualized using chemiluminescence and autoradiography. Densitometry of the phosphorylated Akt and GSK-3 is shown as fold increase (mean OD units control sample/mean OD units experimental sample). *D*, Alveolar macrophages were phosphate-loaded with 1.25 mCi of $^{32}P_i$/group as described in *Materials and Methods*. They were then treated with LPS (1 μg/ml) with and without D609 (10 μM) for 1–60 min. Whole cell protein was obtained and Akt was immunoprecipitated from 500 μg of lysate. A 10% SDS-PAGE gel was run and the phosphorylated protein was visualized by autoradiography.

phosphorylation of ceramide by 1,2-diacylglycerol kinase. The 1,2-diacylglycerol kinase assay showed LPS-induced ceramide increasing as early as 1 min after LPS exposure. To confirm the data using a more sophisticated assay system, we evaluated ceramide levels using HPLC. Alveolar macrophages were treated with LPS, lipids were extracted, and ceramide levels were determined by HPLC. Fig. 3 demonstrates that LPS increases ceramide levels in alveolar macrophages. We evaluated a 30-min time point and believe that these data, in conjunction with the previous study, demonstrate that LPS increases ceramide early and that the increase continues for a substantial period of time. To confirm the effect of PC-PLC on ceramide generation in alveolar macrophages, we also evaluated the generation of ceramide after exogenous PC-PLC (see Fig. 3). PC-PLC also increased alveolar macrophage ceramide. These studies show that LPS exposure generates ceramide and that this can be mimicked by the addition of exogenous PC-PLC.

Ceramide activates PI 3-kinase in alveolar macrophages

Having confirmed that LPS activates the PI 3-kinase pathway and generates ceramide, we next evaluated whether ceramide alone could activate PI 3-kinase. Alveolar macrophages were treated with C2 ceramide, a cell permeable analog, and LPS. Whole cell lysates were obtained, PI 3-kinase was immunoprecipitated, and a kinase activity assay was performed. Fig. 4*A* demonstrates that ceramide activates PI 3-kinase in alveolar macrophages. We confirmed these data by evaluating the tyrosine phosphorylation of p85, the PI 3-kinase regulatory unit, after ceramide (26). Fig. 4*B* demonstrates that ceramide increases tyrosine phosphorylation of p85. The amounts of ceramide used in these experiments are physiologically relevant, as the amounts produced by alveolar macrophages shown in Fig. 3 (20–25 nM/mg protein) convert to ~600 ng/million cells. This is between the 160 ng/million cells (500 nM) and the 3400 ng/million cells (10 μM) used in these experiments. The activation of PI 3-kinase by ceramide is of particular interest because the preponderance of the literature reports systems in which ceramide exposure inhibits PI 3-kinase and triggers apoptosis (37, 38). Fig. 4 shows that ceramide exposure of alveolar macrophages activates PI 3-kinase. These observations suggest that the effects of ceramide on PI 3-kinase signaling may be very different in alveolar macrophages compared with other types of cells.

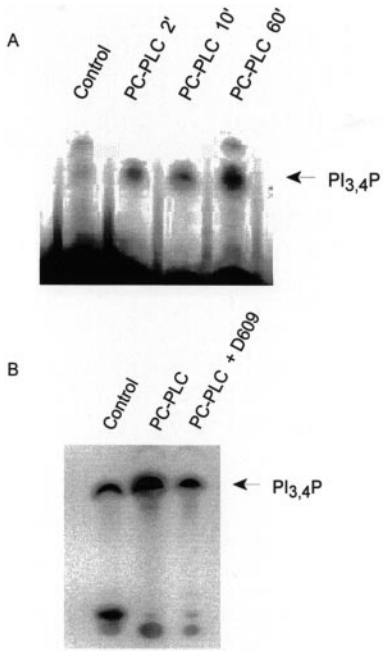

FIGURE 2. Exposure of alveolar macrophages to PC-PLC from *Bacillus cereus* results in activation of PI 3-kinase. Alveolar macrophages were treated with PC-PLC (5 U/ml) for 1–60 min. *A*, Whole cell protein was obtained, p85 was immunoprecipitated, and a kinase activity assay was performed using PI_4P as a substrate. The phosphorylated form was separated on a TLC plate and visualized on a phosphor imager. *B*, The D609 (inhibitor of LPS-induced ceramide) (10 μM) was on for 20 min before the addition of PC-PLC.

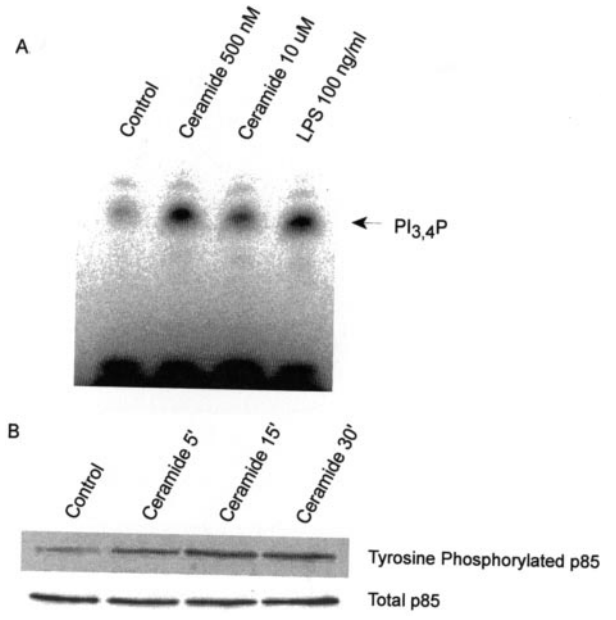

FIGURE 4. Exposure of alveolar macrophages to ceramide activates PI 3-kinase. Alveolar macrophages were treated with ceramide (500 nM or 10 μM) or LPS (100 ng/ml) for 10 min. *A*, Whole cell protein was obtained and PI 3-kinase was immunoprecipitated from 400 μg of the lysates using an Ab specific for the p85 regulatory unit. Kinase activity was determined by evaluating phosphorylation of PI_4P by the immunoprecipitated PI 3-kinase. The resulting $PI_{3,4}P$ was separated in a TLC system and an autoradiogram was obtained. *B*, Whole cell protein was obtained from ceramide-treated alveolar macrophages. PI 3-kinase was immunoprecipitated from 400 μg of the lysates using an Ab specific for the p85 regulatory unit. Western blot analysis was performed and the blot was stained with an Ab specific for phosphorylated tyrosines. Equal loading is demonstrated by staining the blot for total p85.

Ceramide activates Akt and inactivates GSK-3

Alveolar macrophages were treated with ceramide or LPS and whole cell proteins were obtained. Fig. 5*A* shows that ceramide causes increases in phosphorylation of Akt and GSK-3 that are of a magnitude similar to the increases found with LPS. To evaluate the time frame of Akt and GSK-3 phosphorylation by ceramide, we evaluated ceramide signaling between 1 and 60 min. Fig. 5*B* demonstrates a curve of activity beginning as early as 1 min and returning to baseline by 60 min. Variations in the baseline phosphorylations seen in the various experiments in Fig. 5 are explained by the individual variability found among our cell donors. There was some baseline phosphorylation in all of the cells, but the data do demonstrate that LPS and ceramide both increase the amount of Akt and GSK-3 phosphorylation. These observations show that ceramide alone can activate the PI 3-kinase pathway in alveolar macrophages.

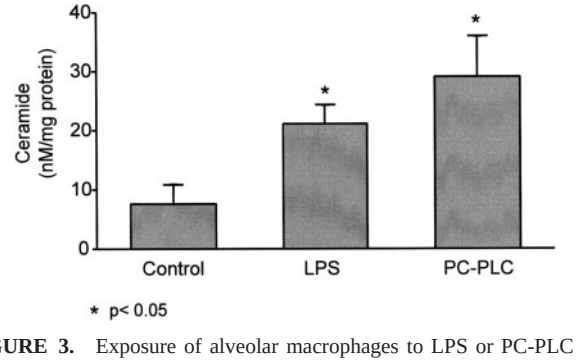

FIGURE 3. Exposure of alveolar macrophages to LPS or PC-PLC results in an increase in ceramide. Alveolar macrophages were treated with LPS (100 ng/ml) or PC-PLC (5 U/ml) for 30 min. Cells were lysed and ceramide amounts were determined by HPLC as described in *Materials and Methods*. The data are presented as nanomoles of ceramide per milligram of protein and is a composite of three separate experiments.

Ceramide phosphorylation of Akt and GSK-3 is PI 3-kinase dependent

There have been recent reports suggesting that other kinases may phosphorylate both Akt and GSK-3 (integrin-linked kinase, protein kinase Cβ, and protein kinase A) (57–59). For this reason it seemed important to confirm that ceramide was activating Akt and inactivating GSK-3 via PI 3-kinase. To do this, alveolar macrophages were treated with the PI 3-kinase inhibitors, wortmannin, and LY294002, followed by ceramide. Fig. 6 shows that the two PI 3-kinase inhibitors blocked ceramide-induced phosphorylation of both Akt and GSK-3. Thus, in alveolar macrophages, ceramide modulates Akt and GSK-3 in a PI 3-kinase-dependent manner.

Ceramide, as compared with LPS, is a poor inducer of NF-κB translocation and DNA binding

To determine whether ceramide exposure activated other antiapoptotic pathways that are activated by LPS, we evaluated the effect of ceramide exposure on NF-κB translocation and DNA binding. NF-κB is a transcription factor known to participate in multiple LPS responses (60). A strong link has been found between NF-κB activity and cell survival (61, 62). We wanted to determine whether ceramide, like LPS, also modulated activation of NF-κB. To do this we treated alveolar macrophages with LPS or ceramide, isolated nuclear protein, and performed an EMSA to determine

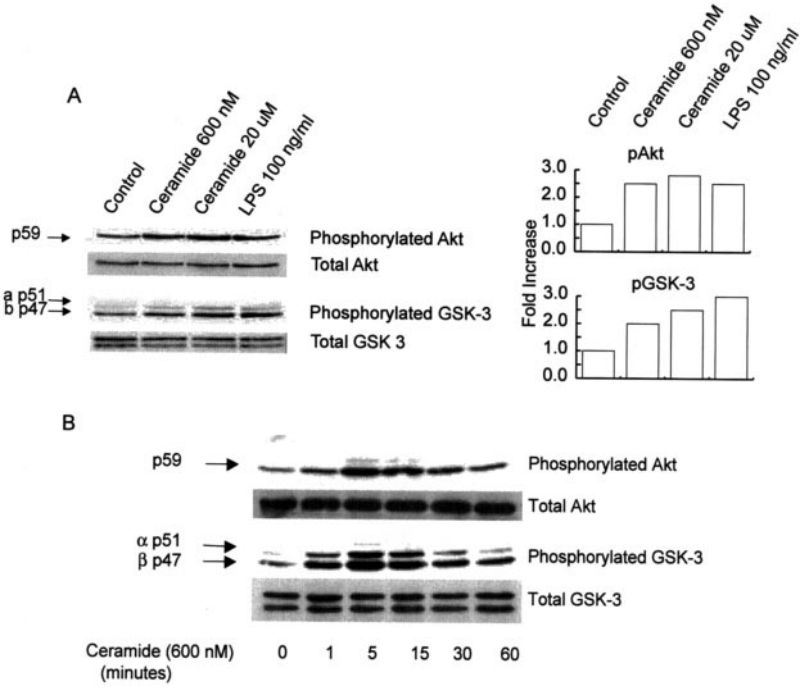

FIGURE 5. Exposure of alveolar macrophages to ceramide results in the phosphorylation of Akt and GSK-3. *A*, Alveolar macrophages were treated with ceramide (500 nM or 10 μM) or LPS (100 ng/ml) for 15 min. Whole cell protein was obtained and Western blot analysis was performed using Abs specific for Akt phosphorylated on serine 473 and GSK-3 phosphorylated on serine 21 in GSK-3 α and serine 9 in GSK-3 β. Equal loading of the blots was demonstrated by stripping the blot and reprobing with an Ab for Akt or GSK-3 (α and β isoforms). Primary Ab concentrations of 1/500 and secondary Ab concentrations of 1/5000 were used. Immunoreactive bands were visualized using chemiluminescence and autoradiography. Densitometry of the phosphorylated Akt and GSK-3 is shown as fold increase (mean OD units 0 time/mean OD units experimental sample). *B*, Alveolar macrophages were treated with ceramide (600 nM) for various times (1–60 min) and phosphorylation of Akt and GSK-3 was evaluated as in *A*.

nuclear translocation and DNA binding (see Fig. 7). We found that LPS caused a strong induction of NF-κB DNA binding and that ceramide exposure resulted in a present but much weaker response. These data suggest that ceramide does not entirely mimic an LPS response, data that are supported by previous comparisons of ceramide and LPS looking at other outcomes (46).

Ceramide activation of PI 3-kinase plays a role in preventing ceramide- and LPS-induced cell death

Ceramide, in most cell systems, triggers cell death by apoptosis (37, 38). We next asked whether the ability of ceramide to activate PI 3-kinase masked its ability to trigger apoptosis. To investigate that hypothesis, we treated alveolar macrophages with the PI 3-kinase inhibitor, LY294002, followed by either ceramide or LPS. The cells were cultured for 24 h and then evaluated for cell viability. Fig. 8 demonstrates that blocking PI 3-kinase activation significantly increases cell death resulting from LPS or ceramide exposure. LY294002 alone had no significant effect on cell viability, but its presence unmasked the proapoptotic effects of LPS and ceramide. Cell death after ceramide was greater than cell death after LPS. This may be at least partially explained by the fact that LPS generates more nuclear DNA-binding NF-κB than does ceramide.

Discussion

This study evaluates the role of LPS-induced ceramide and ceramide alone in activation of the PI 3-kinase pathway in alveolar macrophages. LPS activation of PI 3-kinase and phosphorylation of Akt and GSK-3 were decreased in conditions (D609) that blocked LPS-induced ceramide generation. Ceramide alone activated PI 3-kinase and Akt and inactivated GSK-3 in human alveolar macrophages. Both the activation of Akt and the inactivation of GSK-3 were downstream of PI 3-kinase because they were inhibited by wortmannin and LY294002, PI 3-kinase inhibitors. Because of the preponderance of literature linking ceramide to apoptosis, we evaluated the relationship between ceramide-induced PI 3-kinase activation and ceramide-induced apoptosis in alveolar macrophages. When we blocked the PI 3-kinase pathway with

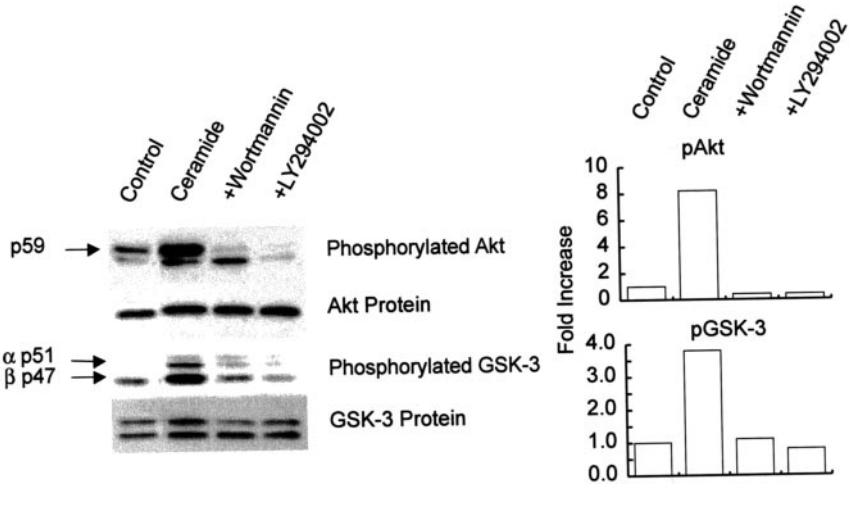

FIGURE 6. Ceramide-induced phosphorylation of Akt and GSK-3 is downstream of PI 3-kinase. Alveolar macrophages were treated with ceramide (600 nM) with and without wortmannin (500 nM) or LY294002 (20 μM) for 15 min. Whole cell protein was obtained and Western blot analysis was performed using Abs specific for Akt phosphorylated on serine 473 and GSK-3 phosphorylated on serine 21 in GSK-3 α and serine 9 in GSK-3 β. Equal loading of the blots was demonstrated by stripping the blot and reprobing with an Ab for Akt or GSK-3 (α and β isoforms). Primary Ab concentrations of 1/500 and secondary Ab concentrations of 1/5000 were used. Immunoreactive bands were visualized using chemiluminescence and autoradiography. Densitometry of the phosphorylated Akt and GSK-3 is shown as fold increase (mean OD units 0 time/mean OD units experimental sample).

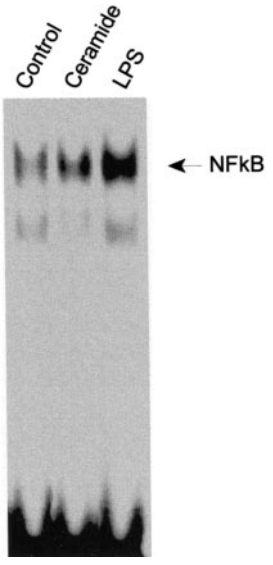

FIGURE 7. Ceramide-induced NF-κB DNA binding is significantly less than that induced by LPS. Alveolar macrophages were treated with ceramide (10 μM) or LPS (100 ng/ml) for 3 h. Nuclear protein was isolated and an EMSA was performed using a probe for the NF-κB consensus sequence labeled with γ-^{32}P. The complex identified as NF-κB could be specifically eliminated with excess unlabeled NF-κB oligonucleotide (data not shown). Visualization was performed on a phosphor imager.

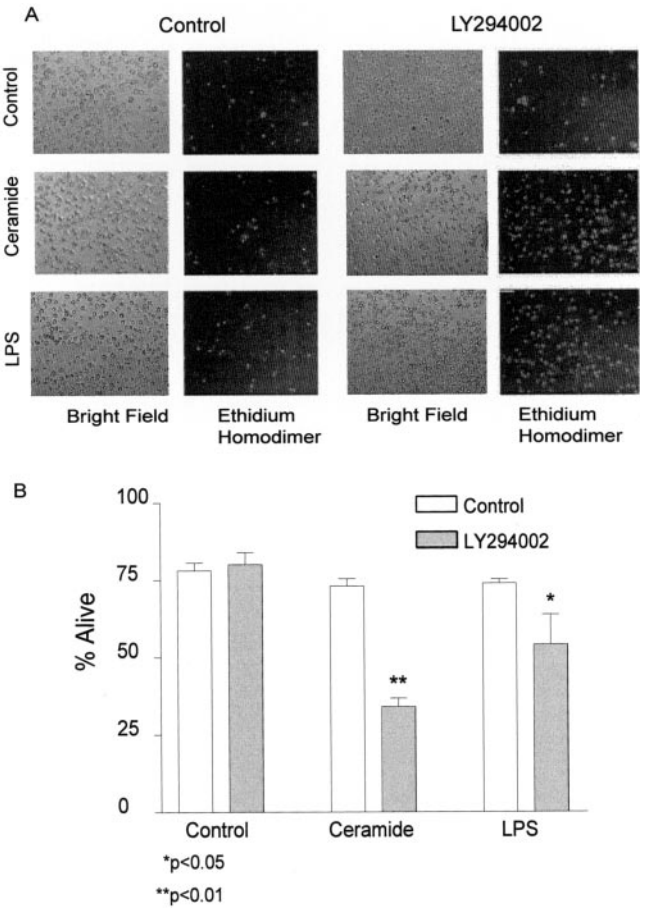

FIGURE 8. Inhibition of PI 3-kinase results in increased cell death of alveolar macrophages after LPS or ceramide exposure. Alveolar macrophages were treated with ceramide (10 μM) or LPS (100 ng/ml) with and without LY294002 (10 μM) and left in culture for 24 h. Cell viability was determined by evaluating the uptake of ethidium homodimer (16 μM). *A*, Photomicrographs demonstrate the nuclear uptake of ethidium homodimer. Dead cells are shown as a white spot and total cells are shown in the bright field exposure. *B*, The graph shows data from three separate experiments analyzed using a fluorescence plate reader. Percentage of live cells is calculated as described in *Materials and Methods.*

LY294002, we found significantly increased cell death of alveolar macrophages after LPS and an even greater cell death after ceramide. The differential effects of ceramide and LPS may be explained by the observation that ceramide was not as efficient as LPS in causing the nuclear translocation of NF-κB. As a composite, these data demonstrate that in alveolar macrophages, the generation of ceramide can serve as a stimulus for activation of PI 3-kinase and downstream mediators. This process appears to mask the cell death-inducing signal of ceramide by promoting cell viability (Fig. 9).

These observations suggest that the generation of ceramide is an important early event in LPS signaling. Potentially, activation of the TLR signaling complex could trigger interaction with a sphingomyelinase. The TNF receptor has been shown to directly bind and activate an adaptor molecule (FAN, factor associated with neutral sphingomyelinase activation) which then activates a neutral sphingomyelinase, resulting in the generation of ceramide (63, 64). Arbibe et al. (47) have shown recently that Gram-positive bacterial stimulation of TLR 2 results in binding of the p85 regulatory unit of PI 3-kinase to TLR 2. They suggest a model in which PI 3-kinase and Rac1 are directly activated by TLR 2 in a MyD88-independent manner. TLR 4 lacks the p85 binding motif that is present in TLR 2, but other possibilities exist (MyD88, IL-1R accessory protein) that might link LPS-induced PI 3-kinase activation to the TLR. Recently, Horng et al. (65) have described the existence of a new adaptor protein at the TLR 4 complex, TIRAP. TIRAP is an adaptor molecule that controls MyD88-independent signaling from the TLR 4 complex. This is another possible link between the TLR 4 complex and PI 3-kinase activation. If this is the case, it follows that LPS-induced ceramide generation probably also occurs at the level of the TLR complex. Several models

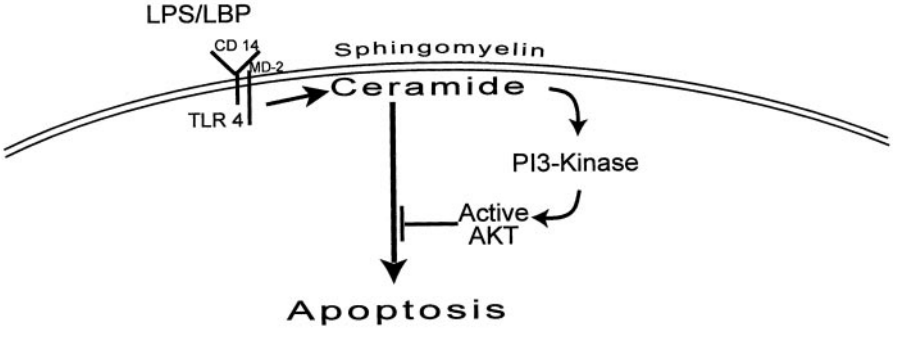

FIGURE 9. This is a diagrammatic representation of how the data presented in this study fit together. LPS-induced ceramide in alveolar macrophages results in activation of the PI 3-kinase pathway, preventing ceramide-induced apoptosis.

exist for ceramide generation at either the outer or inner plasma membranes (38). Either site would allow for interactions with the TLR (2 or 4) itself or with membrane-recruited PI 3-kinase.

Our data that LPS-induced ceramide does not induce cell death at 24 h contrast with other observations that ceramide induces cell death (66–70). Zundel and Giaccia (71) have shown that in Rat-1 fibroblasts, ceramide inhibits PI 3-kinase by sequestering the p85 regulatory unit with caveolin-1. In neuronal cells, addition of C2 ceramide blocks both basal and stimulated Akt activity (72). Schubert and Duronio (73) showed, in TF-1 erythroleukemia cells, that ceramide directly accelerated the dephosphorylation of Akt at serine 473 (a phosphorylation required for activation of the kinase). Ceramide has been strongly linked to inhibition of insulin-activated PI 3-kinase and Akt (20, 74). In an animal model of sepsis, acid sphingomyelinase knockout animals were protected from endothelial apoptosis and cell death, suggesting that in this model decreased ceramide led to decreased cell death (75).

Macrophages and ceramide have been studied by S. Vogel's group (76), who found that macrophages from mice with defective TLR 4 lacked both LPS and ceramide responses, suggesting that both required TLR 4. This would support our contention that LPS-generated ceramide is intimately involved with the TLR complex. This group also found ceramide induced cell death in murine peritoneal macrophages. In that study, they used thioglycolate-elicited murine peritoneal macrophages and treated them with 25 μM C2 ceramide. This resulted in significant cell death at 24 h (77). These data can be reconciled with our data because in human alveolar macrophages both LPS and ceramide activate the PI 3-kinase pathway, which potently inhibits apoptosis. When we inhibited the PI 3-kinase pathway, both ceramide and LPS triggered apoptosis, consistent with prior studies. Thus, the key difference in these studies is the activation, not inhibition, of PI 3-kinase by ceramide exposure in a different macrophage population, human alveolar macrophages.

Alveolar macrophages are a subpopulation of cells that plays a primary role in host defense. They are characterized by distinct surface markers (78) and by distinct and unique signaling pathways (49, 79). Alveolar macrophages have been shown to survive for extended times after a foreign insult (80, 81). Because the lung is often the first site of exposure to environmental stimuli, it is logical that alveolar macrophages have developed a unique method of prolonging and modulating inflammatory responses. We believe that LPS generation of ceramide plays a role in survival of the cells, which is necessary for mediating a prolonged and effective inflammatory state.

This is not the first study to show that ceramide can activate PI 3-kinase and Akt. Human colonic smooth muscle cells have been shown to have PI 3-kinase activation downstream of ceramide (72, 82). The other significant study demonstrating ceramide activation of PI 3-kinase is that by Hanna and Brindley (54), demonstrating that TNF-induced ceramide in fibroblasts activated PI 3-kinase via tyrosine kinase activity and $p21^{ras}$. However, our study is the first one to link ceramide to PI 3-kinase activation in the setting of LPS-activated alveolar macrophages. It is also the first study to demonstrate that, in some settings (alveolar macrophages), ceramide can contribute to cell viability.

Akt inhibits apoptosis by a diverse array of stimuli (UV light, matrix detachment, DNA damage, CD95 ligation, and viral infection) (17). Multiple Akt effectors are capable of mediating this response: 1) phosphorylation and inactivation of GSK-3 results in the accumulation and transcriptional activity of β catenin-driven progrowth genes, 2) phosphorylation of Bad causes its sequestration with 14-3-3, protecting the mitochondria, and 3) phosphorylation of caspase 9 inhibits its activity and subsequent activation of effector kinases (29, 58). These Akt-dependent events likely are responsible for the inhibition of apoptosis in human alveolar macrophages when ceramide activates PI 3-kinase and Akt.

These observations, as a whole, suggest that human alveolar macrophages possess a mechanism to prolong cell viability after exposure to endotoxin and/or ceramide. This may be explained by the unique ability of these cells to use ceramide as an activator of PI 3-kinase and Akt.

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